

Functional genetic validation of key genes conferring insecticide resistance in the major African malaria vector, *Anopheles gambiae*

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Resistance in *Anopheles gambiae* to members of all 4 major classes (pyrethroids, carbamates, organochlorines, and organophosphates) of public health insecticides limits effective control of malaria transmission in Africa. Increase in expression of detoxifying enzymes has been associated with insecticide resistance, but their direct functional validation in *An. gambiae* is still lacking. Here, we perform transgenic analysis using the GAL4/UAS system to examine insecticide resistance phenotypes conferred by increased expression of the 3 genes—*Cyp6m2*, *Cyp6p3*, and *Gste2*—most often found up-regulated in resistant *An. gambiae*. We report evidence in *An. gambiae* that organophosphate and organochlorine resistance is conferred by overexpression of GSTE2 in a broad tissue profile. Pyrethroid and carbamate resistance is bestowed by similar *Cyp6p3* overexpression, and *Cyp6m2* confers only pyrethroid resistance when overexpressed in the same tissues. Conversely, such *Cyp6m2* overexpression increases susceptibility to the organophosphate malathion, presumably due to conversion to the more toxic metabolite, malaoxon. No resistant phenotypes are conferred when either *Cyp6* gene overexpression is restricted to the midgut or oenocytes, indicating that neither tissue is involved in insecticide resistance mediated by the candidate P450s examined. Validation of genes conferring resistance provides markers to guide control strategies, and the observed negative cross-resistance due to *Cyp6m2* gives credence to proposed dual-insecticide strategies to overcome pyrethroid resistance. These transgenic *An. gambiae*-resistant lines are being used to test the “resistance-breaking” efficacy of active compounds early in their development.

functional analysis | cytochromes P450 | glutathione-S-transferase | GAL4/UAS

The number of worldwide malaria cases reduced steadily from 2000 until 2015, mainly due to the widespread rollout of insecticide-treated bed nets in endemic areas (1), which offer protection against bites from *Plasmodium*-infected *Anopheles* mosquitoes. Since then, the drop in malaria cases has stalled (2), which has been attributed partially to the increasing levels of insecticide resistance found in *Anopheles* vectors (3). Resistance in dominant African *Anopheles* vectors has been recorded to all major insecticide classes currently used in public health (pyrethroids, organochlorines, carbamates, and organophosphates [OPs]) (4). Therefore, understanding the mechanisms by which mosquitoes evolve resistance is critical for the design of mitigation strategies and in the evaluation of new classes of insecticides.

Research into the molecular mechanisms that give rise to resistance in mosquitoes has identified target site modifications and increased metabolic detoxification (detox) as the 2 main evolutionary adaptations (5) that often coexist in *Anopheles gambiae*. Families of detoxification enzymes, including cytochromes P450 (CYPs) and glutathione-S-transferases (GSTs), can provide phase I metabolism of insecticides and phase II conjugation reactions that alter the toxicity of compounds and increase polarity, enhancing excretion (6, 7).

To identify and characterize the role of the causative resistance genes from these detoxification families, a sequential process

of transcriptomic, proteomic, and in vivo functional analysis is often applied (8). Candidate genes with up-regulated transcription or strong signatures of selection in resistant mosquitoes are typically expressed in bacteria to provide evidence of insecticide depletion and/or metabolism in vitro (9–19). Further studies have used the *Drosophila melanogaster* transgenic model to determine whether expression of single *Anopheles* genes confers increased tolerance to insecticides (13–18, 20).

This workflow has implicated a role in resistance of 2 CYP genes, *Cyp6m2* and *Cyp6p3*, and a GST gene, *Gste2*, that are consistently up-regulated in resistant field populations found across Africa (21). However, there are often discrepancies in results from recombinant protein activity and transgenic *D. melanogaster* analyses. For example, while expression studies of *Cyp6m2* and *Cyp6p3* in *Escherichia coli* (10, 11) and *D. melanogaster* (15) suggest that both gene products can detoxify pyrethroids, the 2 systems produce conflicting results in respect to carbamate (15) and organochlorine insecticide detoxification (12, 15, 19). Moreover, the involvement of *An. gambiae* and *Anopheles funestus* *Gste2* (*AfGste2*) orthologs in

Significance

Insecticide resistance in *Anopheles gambiae* mosquitoes can derail malaria control programs, and to overcome it, we need to discover the underlying molecular basis. Here, we characterize 3 genes most often associated with insecticide resistance directly by their overproduction in genetically modified *An. gambiae*. We show that overexpression of each gene confers resistance to representatives of at least 1 insecticide class, and taken together, the 3 genes provide cross-resistance to all 4 major insecticide classes currently used in public health. These data validate the candidate genes as markers to monitor the spread of resistance in mosquito populations. The modified mosquitoes produced are also valuable tools to prescreen the efficacy of new insecticides against existing resistance mechanisms.

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The authors declare no competing interest.

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Data deposition: We have supplied all of the raw insecticide bioassay data at Figshare, https://figshare.com/articles/Raw_data_Bioassays.xlsx/10000388/1.

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resistance to pyrethroid insecticides has produced contradictory results when explored in *D. melanogaster* (16, 20).

Clearly, functional validation of *Anopheles* genes directly in the mosquito would provide the benchmark approach to address these questions; however, to date, transgenic tools to perform such analysis have been limited. To this end, we have developed the GAL4/UAS expression system in *An. gambiae* (22–24), which allows genes to be overexpressed in a susceptible mosquito background and for resultant resistance phenotypes to be examined using the standard insecticide assays that have been developed for comparative analysis in mosquitoes by the World Health Organization (WHO) (25).

In vivo functional analysis in *Anopheles* can also help discover the mosquito tissues that are specifically involved in insecticide metabolism. Our previous research indicated high P450 activity in the midgut and oenocytes, since the essential P450 coenzyme, cytochrome P450 reductase (CPR), is highly expressed in these tissues, and RNA interference (RNAi) knockdown of *Cpr* increased mosquito sensitivity to a pyrethroid insecticide (26). Moreover, *Cyp6m2* has been reported as enriched in the *An. gambiae* midgut (11), and *Cyp6p3* was found up-regulated in midguts from pyrethroid-resistant populations (27).

Here, we have used the GAL4/UAS system to overexpress *Cyp6m2* or *Cyp6p3* genes in multiple tissues or specifically in the midgut or oenocytes of a susceptible *An. gambiae* strain and assayed the modified mosquitoes against representatives of each insecticide class available for public health use. In doing so, we determined the resistance profile generated for each gene and compared these results with those obtained in *D. melanogaster* and in vitro. We then analyzed the other major candidate, *Gste2*, to examine its role in conferring dichloro-diphenyl-trichloro-ethane (DDT) resistance and also, extended its testing to other classes of insecticides in which its role has yet to be tested in vivo.

In this work, we report the use of the GAL4/UAS system in *An. gambiae* as a benchmark to determine whether single candidate genes and/or expression in individual tissues are able to confer WHO-defined levels of resistance to the 4 public health classes of insecticides, including OPs. Crucially we find that, when assayed in *An. gambiae*, overexpression of *Cyp6m2*, *Cyp6p3*, or *Gste2* produces

cross-resistance phenotypes that encompass members of all 4 classes of insecticides currently used for malaria control.

Results

Mosquito Lines Generated for UAS-Regulated Expression of *Cyp6m2* and *Cyp6p3*. YFP-marked UAS-*Cyp6m2* and -*Cyp6p3* lines were created by site-directed recombination-mediated cassette exchange (RMCE) into the docking (CFP:2xattP) line A11 (24) to produce mosquitoes carrying transgene insertions in the same genomic site. By normalizing potential genomic position effect, this allows more reliable comparison of the consequences of *Cyp6m2* and *Cyp6p3* overexpression on resistance.

A summary of the screening and crossing strategy used to create the UAS responder lines is illustrated in Table 1. RMCE results in canonical cassette exchange in 2 potential orientations; however, integration of the whole donor transgene can also occur in either attP site. Fluorescent marker screening of F₁ progenies from F₀ pooled mosquitoes revealed that cassette exchange and integration events occurred in all experiments as shown by the recovery of individuals carrying single (YFP: exchange) or double (YFP/CFP: integration) markers (Table 1).

Molecular analysis revealed one exchange orientation (A) in transgenic UAS-m2 individuals and both orientations for UAS-p3 transformation as indicated by diagnostic PCR (SI Appendix, Fig. S1). Overall, we found at least 2 events for UAS-m2 transformation, having equal efficiencies of 2% for cassette exchange and integration (1/49 F₀ founders); for the UAS-p3 transformation, at least 9 transformation events (6 cassette exchanges, 3 in each orientation, and 3 transgene integrations) were detected, with a minimum cassette exchange efficiency of 5% (6/124 F₀) and integration efficiency of 2% (3/124 F₀). For comparative functional analysis, representative *Cyp6* lines in orientation A were maintained and crossed with alternative GAL4 driver lines.

CYP6M2 or CYP6P3 Overexpression in Multiple Tissues Causes Distinct Profiles of Resistance to Pyrethroids and Bendiocarb. We previously described the production of a GAL4 driver line, Ubi-A10, directing widespread tissue expression (23). To quantify the overexpression achieved with this driver, we performed qRT-PCR in the progeny of

Table 1. Summary of the screening and crossing strategy adopted to create and establish the UAS responder lines by RMCE

| Docking line (no. of embryos) and F ₀ pools (no. and sex) | F ₀ isofemale | F ₁ transgenics | | Orientation of cassette exchange* |
|--|--------------------------|----------------------------|------------------|---|
| | | YFP+ | YFP+/CFP+ | |
| A11_UAS- <i>Cyp6m2</i> (347) | | | | |
| M2-1 (24 ♀) | G | 0 | 2 | N/A |
| | J | 2♂ | 0 | 2 F ₁ ♂-A |
| M2-2 (25 ♂) | N/A | 0 | 0 | N/A |
| A11_UAS- <i>Cyp6p3</i> (460) | | | | |
| P3-1 (28 ♀) | N/A | 7♀, 4♂ | 1 | 5 F ₁ ♀-A ×2, B ×3 |
| P3-2 (27 ♀) | N/A | 2♀, 8♂ | 2 | 2 F ₁ ♀-A, B |
| P3-3 (13 ♀) | N/A | 0 | 0 | N/A |
| P3-4 (56 ♂) | N/A | 10♀, 13♂ | 4 | 3 F ₁ ♀-A, B ×2 |
| Ubi-A10_UAS- <i>Gste2</i> (208) | | | | |
| E2-1 (10 ♂) | N/A | 0 | 0 | N/A |
| E2-2 (12 ♀) | N/A | 0 | 0 | N/A |
| E2-3 (19 ♂) | N/A | 2♂ | 36♀, 44 ♂ | 2 F ₁ ♂-A |
| E2-4 (24 ♀) | A | 3♀, 3♂ | (7) [†] | F ₂ progeny of 1 F ₁ ♂-B |
| | E | 4♀, 3 ♂ | 2♀, 2♂ | 1 F ₁ ♀-A F ₂ progeny of 1 F ₁ ♂-A |

Numbers in brackets after the docking line names in column 1 refer to number of eggs injected. M2, P3, and E2 refer to pools of single sex F₀ adults (number of mosquitoes given in brackets) collected post injection that gave rise to F₁ progeny. G, J, A, and E refer to F₀ females laying eggs individually that gave rise to isofemale lines. N/A, not applicable. YFP+ and YFP+/CFP+ indicate the number and sex of F₁ progeny showing YFP or YFP and CFP fluorescence, respectively.

*As cassette exchange may occur in 2 different orientations with respect to the chromosome, designated A or B, orientation check was performed on F₁ YFP+ individuals or on the F₂ progeny derived from single YFP+ individuals.

[†]Did not survive to adulthood.

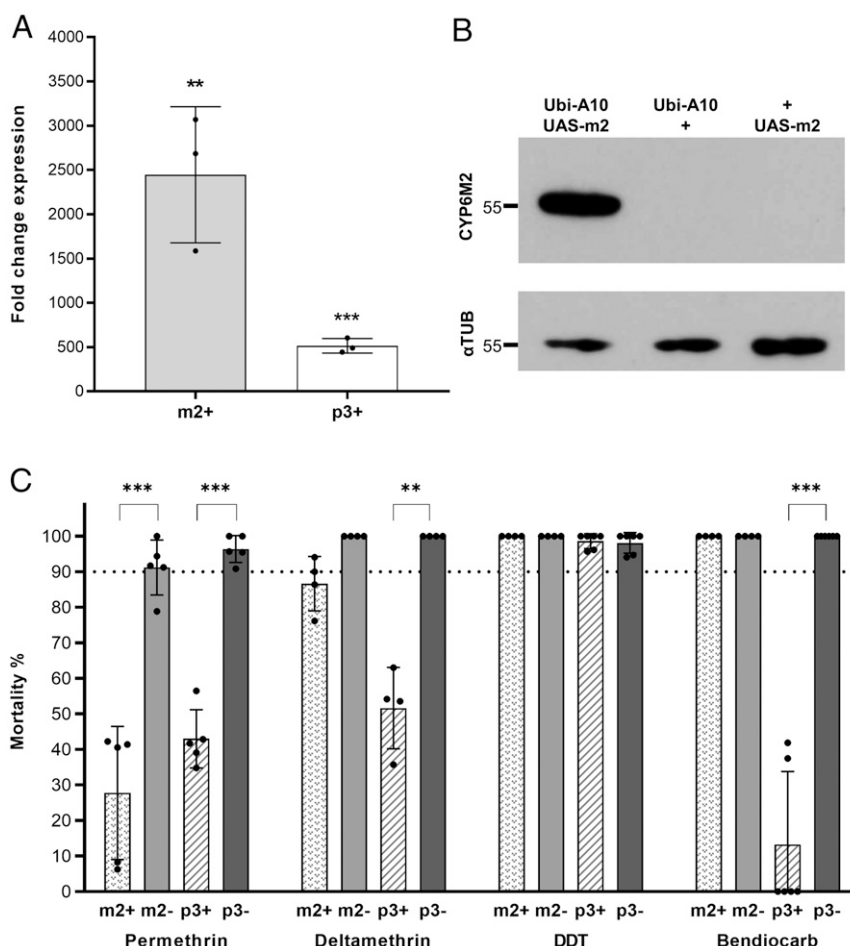


Fig. 1. Multitissue *Cyp6* gene up-regulation affects sensitivity to 2 pyrethroids and a carbamate insecticide. (A) Relative transcription levels of *Cyp6m2* (m2+) and *Cyp6p3* (p3+) in adult females where expression is driven by the Ubi-A10 driver compared with *GAL4/+* controls. Bars represent SD ($n = 3$). Unpaired *t* test. $^{***}P < 0.001$; $^{**}P < 0.01$. (B) Expression of CYP6M2 and α -tubulin in adult females from Ubi-A10 \times UAS-m2 crosses with respective Ubi-A10/+ and +/UAS-m2 controls. Protein extract from the equivalent of 1/10 of a whole female mosquito was loaded in each lane. (C) Sensitivity to insecticides of *GAL4/UAS* (+) females overexpressing *Cyp6m2* or *Cyp6p3* ubiquitously under the control of the Ubi-A10 driver compared with *GAL4/+* controls (–) measured by WHO tube bioassay. Bars represent SD ($n = 4$ to 6) (SI Appendix, Table S2). The dotted line marks the WHO 90% mortality threshold for defining resistance. Welch's *t* test with $P < 0.01$ significance cutoff. $^{**}P < 0.01$; $^{***}P < 0.001$.

Ubi-A10 driver and UAS-*Cyp6* crosses. This revealed significant 2,447 \times ($P = 0.005$) and 513 \times ($P < 0.001$) increases of *Cyp6m2* and *Cyp6p3* transcript abundance, respectively, in adult females compared with native expression in respective controls (Fig. 1A). Western analysis also readily detected CYP6M2 in the adult female progeny of the Ubi-A10/UAS-m2 crosses but was beyond the level of detection in sibling controls (Ubi-A10/+ and +/UAS-m2) (Fig. 1B). No suitable antiserum was available for analysis of CYP6P3.

WHO discriminating dose assays were then performed to assess the susceptibility of mosquitoes overexpressing *Cyp6m2* or *Cyp6p3* compared with their Ubi-A10/+ siblings. WHO tube bioassays are used to screen for the emergence of resistance in field populations and involve exposing mosquitoes to fixed concentration of insecticides (twice the lethal concentration that kills 99% of a susceptible population) for 60 min followed by a 24-h recovery period before recording mortality (25). The parental strains used here are susceptible ($>90\%$ mortality) to all of the insecticides tested; therefore, a decrease in mortality in test assays can be directly attributable to the overexpression of the specific candidate gene.

Mosquitoes overexpressing either *Cyp6* gene under the Ubi-A10 driver showed resistance to permethrin (*Cyp6m2*: 28% mortality, $P < 0.001$; *Cyp6p3*: 43% mortality, $P < 0.001$) and deltamethrin (*Cyp6m2*: 88%, $P = 0.04$; *Cyp6p3*: 52%, $P = 0.004$) compared with controls (Fig. 1C). A significant difference in mortality was

observed between mosquitoes overexpressing the 2 different *Cyp6* genes for deltamethrin assays ($P = 0.003$), while no significant difference was observed for permethrin ($P = 0.15$). However, only *Cyp6p3*-overexpressing mosquitoes showed resistance to bendiocarb (13% mortality, $P < 0.001$) (Fig. 1C). No resistance to DDT was observed with either gene in conjunction with the Ubi-A10 driver (Fig. 1C).

CYP6M2 or CYP6P3 Multitissue Overexpression Increases Susceptibility to Malathion. Malathion is an OP proinsecticide that is activated in vivo to the more toxic compound malaoxon through P450-based oxidative reactions (28). Preliminary analysis showed that a standard WHO diagnostic dose and 60-min exposure killed all test and control mosquitoes; however, during exposure, it was clear that Ubi-A10-directed *Cyp6* overexpression induced more rapid knockdown compared with controls, suggesting malathion activation by these P450s. We, therefore, examined the relative sensitivity of mosquitoes overexpressing *Cyp6m2* or *Cyp6p3* when exposed to the same diagnostic dose of this OP for a shorter time (25 min) (Fig. 2). Under these conditions, mosquitoes overexpressing *Cyp6m2* under the control of the Ubi-A10 driver showed significantly higher mortality rates compared with controls (95 vs. 15%, $P < 0.001$) and Ubi-A10/UAS-p3 mosquitoes (95 vs. 34%, $P = 0.002$), although the

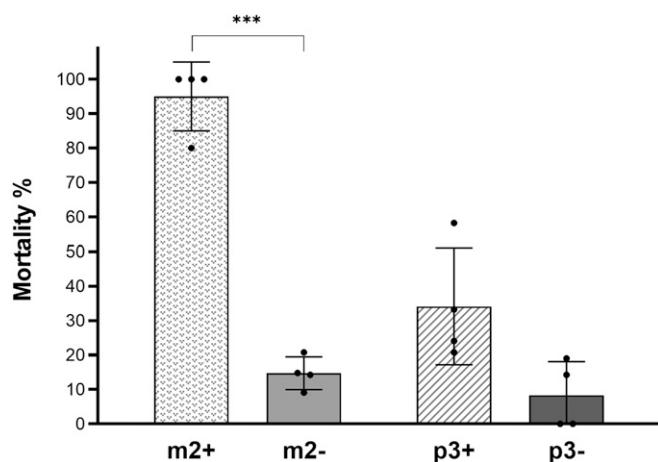


Fig. 2. Multitissue *Cyp6* gene up-regulation increases sensitivity to the OP insecticide malathion (25-min exposure). Sensitivity to malathion of females overexpressing *Cyp6m2* (m2+) or *Cyp6p3* (p3+) ubiquitously under the control of the Ubi-A10 driver compared with respective GAL4/+ controls (m2-, p3-) measure by a modified WHO tube bioassay representing mortality rates after 25 min of exposure and 24-h recovery. Bars represent SD ($n = 4$) (SI Appendix, Table S2). Welch's t test with $P < 0.01$ significance cutoff. *** $P < 0.001$.

latter also showed a trend of increased mortality compared with Ubi-A10/+ controls (34 vs. 8%, $P = 0.05$).

Overexpression of GSTE2 in Multiple Tissues Causes Resistance to Diagnostic Doses of DDT and Fenitrothion. To extend the analysis to the role of GSTE2 in insecticide resistance in *An. gambiae*, we utilized the previously described Ubi-A10 GAL4 line (23) as a docking line. Integration of the UAS cassette into a single docking site in this case would provide Ubi-A10GAL4 and UAS-*Gste2* at the same locus (Ubi-A10GAL4:UAS-e2) and should natively overexpress *Gste2* without the need for crossing separate lines. Alternatively, cassette exchange would generate a regular UAS-*Gste2* responder line. After embryonic injections and screening, 3 exchange events (2 in orientation A and 1 in orientation B) (SI Appendix, Fig. S1) and 3 integration events were independently recovered with an overall transformation efficiency

of 9% (6/65 F_0), exchange efficiency of 5% (3/65 F_0), and integration efficiency of 5% (3/65 F_0) (Table 1).

To obtain comparable data for *Gste2* and the *Cyp6* genes, we focused our analysis on the progeny from crosses between UAS-e2 and Ubi-A10GAL4 mosquitoes. When exposed to diagnostic doses of DDT, GSTE2-overexpressing mosquitoes showed a significantly lower mortality (7%, $P < 0.001$) compared with controls, while no significant difference in resistance was found when exposed to diagnostic doses of permethrin, deltamethrin, malathion, or bendiocarb (Fig. 3). A trend of increased tolerance was observed in mosquitoes overexpressing *Gste2* against malathion (Fig. 3), and further analysis with the related OP fenitrothion indicated high resistance in Ubi-A10/UAS-e2 mosquitoes, showing 8% ($P < 0.001$) mortality (Fig. 3).

Preliminary analysis of Ubi-A10GAL4:UAS-e2 (integration) mosquitoes indicated the expected increase in GSTE2 protein in whole-body extracts compared with Ubi-A10 controls (SI Appendix, Fig. S2A) and a resistance phenotype against DDT in the F_1 generation of transformed male and female mosquitoes (SI Appendix, Fig. S2B).

Oenocyte or Midgut-Specific Overexpression of CYP6M2 or CYP6P3 Does Not Confer Resistance to Insecticides.

To examine the role of oenocytes and midgut tissues in P450-based metabolism of insecticides, we utilized previously published GAL4 driver lines to regulate tissue-specific expression. The specificity of these GAL4 lines to drive the respective tissue-specific expression has been established following crosses with UAS-regulated fluorescent gene reporter lines (22, 24). To examine the relative increase in *Cyp6* gene expression, we performed qRT-PCR and western blot analysis in dissected body parts from progeny of alternative driver and *Cyp6* responder crosses. Using the midgut driver (GAL4-mid), *Cyp6m2* and *Cyp6p3* transcripts were 2,730 \times ($P = 0.002$) and 659 \times ($P = 0.011$) more abundant, respectively, in midguts dissected from GAL4/UAS mosquitoes compared with controls (Fig. 4A). A low level of overexpression was detected in the remaining carcass of GAL4/UAS mosquitoes compared with that of controls (*Cyp6m2*: 77 \times , $P = 0.038$; *Cyp6p3*: 7 \times , $P = 0.08$). In GAL4-oenocrosses, *Cyp6m2* and *Cyp6p3* were specifically up-regulated in transgenic dissected abdomens (66 \times , $P = 0.013$ for *Cyp6m2*; 153 \times , $P < 0.001$ for *Cyp6p3*) where oenocytes are located (Fig. 4B). Background overexpression was also found in the remaining carcass of GAL4/UAS-m2 and -p3 adults compared with controls

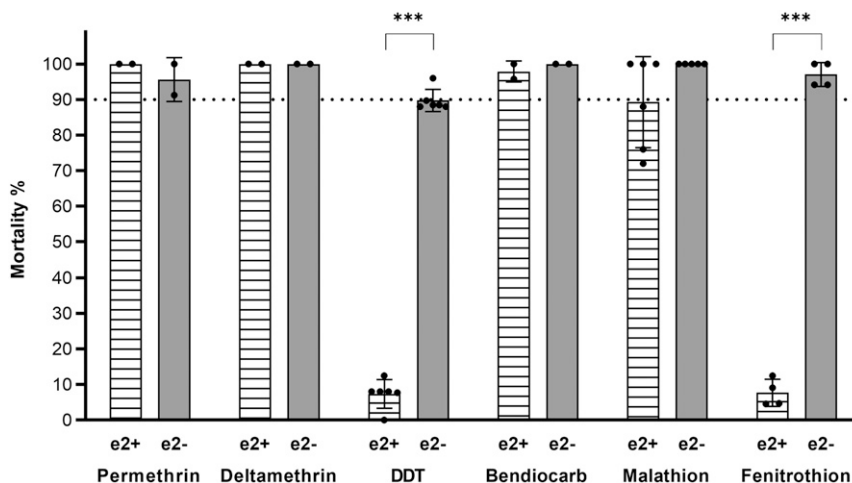


Fig. 3. Multitissue overexpression of GSTE2 affects sensitivity to an organochlorine and an OP insecticide. Sensitivity to insecticides of adult female mosquitoes overexpressing *Gste2* (e2+) ubiquitously under the control of the Ubi-A10 driver compared with Ubi-A10 controls (e2-) measured by WHO tube bioassay. Bars represent SD ($n = 2$ to 6) (SI Appendix, Table S2). The dotted line marks the WHO 90% mortality threshold for defining resistance. Welch's t test with $P < 0.01$ significance cutoff. *** $P < 0.001$.

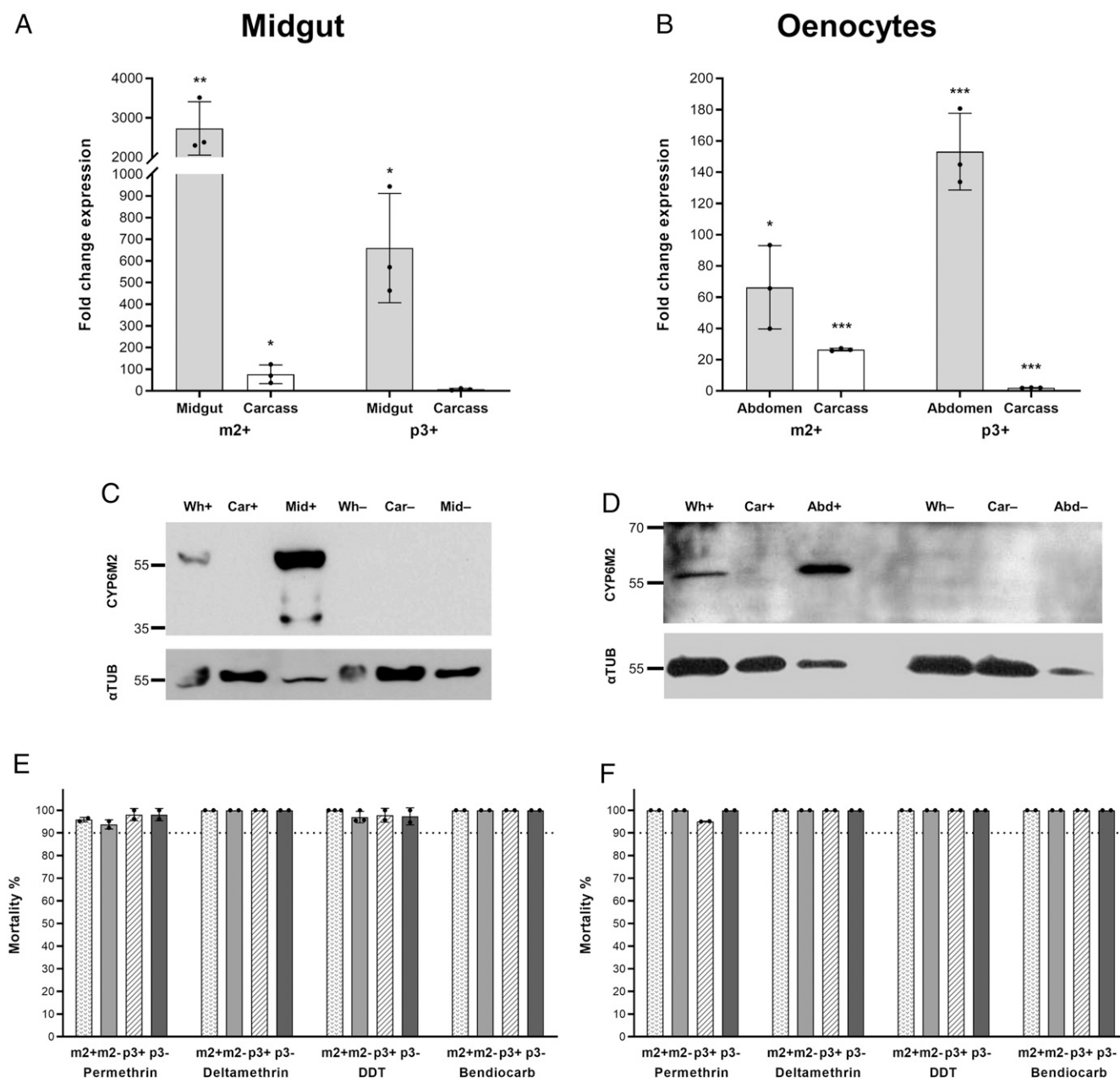


Fig. 4. *Cyp6* gene up-regulation in the mosquito midgut or oenocytes does not affect sensitivity to insecticides. (A and B) Relative transcription levels of *Cyp6m2* (m2+) and *Cyp6p3* (p3+) in dissected midguts (A) and abdomens (B) of GAL4/UAS female mosquitoes compared with the equivalent body parts in GAL4/+ controls. Carcass is whole body without the relevant dissected part. Bars represent SD ($n = 3$). Unpaired t test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C and D) Expression of CYP6M2 and α -tubulin in females from the GAL4-mid \times UAS-m2 (C) and GAL4-oen \times UAS-m2 (D) crosses. Abd, abdomen cuticle; Car, protein extract from 1/3 of a single female carcass-whole body without the midgut (C) or the abdomen (D); Mid, 2 dissected midguts; Wh, protein extract from 1/3 of a single whole female. +, GAL4/UAS-m2; –, GAL4/+. (E and F) Sensitivity to insecticides of GAL4/UAS females overexpressing (+) *Cyp6m2* or *Cyp6p3* in the midgut (E) or in the oenocytes (F) compared with GAL4/+ controls (–) measured by WHO tube bioassay. Bars represent SD ($n = 2$ to 3) (SI Appendix, Table S2). Dotted lines mark the WHO 90% mortality threshold for defining resistance. Welch's t test with $P < 0.01$ significance cutoff.

(26 \times , $P < 0.001$ and 2 \times , $P < 0.001$, respectively). In western blot analysis, CYP6M2 antiserum again only detected the target protein in GAL4/UAS mosquitoes. CYP6M2 was found exclusively in dissected midguts (and whole mosquitoes) from the progeny of GAL4 midcrosses but was not observed in GAL4/UAS carcasses or extracts from controls (Fig. 4C). Similarly, in GAL4-oen crosses, CYP6M2 signal was only detected in whole adult female extracts and in dissected abdomen integument but not in the remaining carcass or control extracts (Fig. 4D).

Adult females overexpressing *Cyp6m2* in the midgut (Fig. 4E) or in the oenocytes (Fig. 4F) showed complete susceptibility to permethrin, deltamethrin, DDT, and bendiocarb. Similar results were obtained with *Cyp6p3* (Fig. 4E and F); however, potential resistance (95% mortality, $P = 0.013$) was suggested in oenocyte-specific *Cyp6p3*-overexpressing mosquitoes when exposed to permethrin (Fig. 4F). Further analysis was performed to detect subtle differences in susceptibility by repeating the assays with reduced exposure time (SI Appendix, Fig. S3). However, no significant decrease ($P < 0.01$)

was found in the mortality rates of mosquitoes overexpressing *Cyp6m2* or *Cyp6p3* in the midgut or oenocytes compared with their respective controls when exposed for 20 min to the same diagnostic doses of the 4 insecticides (SI Appendix, Fig. S3).

Finally, the 25-min reduced exposure bioassay for malathion showed no significant difference in the mortality of mosquitoes overexpressing *Cyp6m2* or *Cyp6p3* in midgut or oenocytes compared with controls (SI Appendix, Fig. S4).

Discussion

In vivo functional analysis is critical to provide evidence of causative links between candidate genes and their proposed phenotypes. Here, we demonstrate the utility of GAL4/UAS-based tools to characterize gene function directly in *An. gambiae* by reporting the use of the system to validate the ability of single candidate genes to confer WHO-defined resistance to different classes of insecticides. Overall, the transgenic analysis in *An. gambiae* is more in accordance with data generated from recombinant protein studies of insecticide metabolism rather than those obtained from *D. melanogaster* survival assays (Table 2).

In *Anopheles*, multitissue overexpression of *Cyp6m2* and *Cyp6p3* demonstrated that resistance to permethrin and deltamethrin (types I and II pyrethroids, respectively) can be conferred by the sole overexpression of either *Cyp6* gene. *Cyp6p3* expression also conferred resistance to bendiocarb (carbamate), while the overexpression of either *Cyp6* gene did not alter DDT (organochlorine) sensitivity. These phenotypes correlate with the profile of metabolism or substrate depletion of the respective

insecticides for the 2 recombinant P450 enzymes (Table 2). More variable results have been observed using *D. melanogaster* as an in vivo model, with overexpression of *Cyp6m2* surprisingly generating increased tolerance to bendiocarb compared with *Cyp6p3*, despite in vitro analysis not detecting activity against bendiocarb for *Cyp6m2* (15, 19). DDT tolerance was also observed in *Cyp6m2*-overexpressing *D. melanogaster*, but data for *Cyp6p3* could not be generated (15) (Table 2). In this study, DDT resistance was monitored by dose–response assays over a 24-h exposure time, while bendiocarb resistance was not observed when measured through such dose–response assays but was reported following 24-h exposure to a diagnostic dose. In the latter case, the controls used to compare *Cyp6m2* and *Cyp6p3* overexpression showed very different levels of sensitivity to bendiocarb, which seemed to contribute to the differences in resistance levels observed, while there were no data for the respective *Cyp6p3* controls in the DDT analysis for comparison. It may thus be differences in the insecticide susceptibility of the control lines that give rise to the discrepant results observed in *D. melanogaster*. Since the *D. melanogaster* UAS-*Cyp6* lines were also created by PhiC31 transformation, it seems unlikely that the differences are caused by position effects. However, it should also be noted that the different methods of insecticide bioassay performed in the *D. melanogaster* studies may not yield directly comparable results with the diagnostic WHO level of resistance in mosquitoes used in this study and extensively used to assess the emergence of resistance in endemic countries. Our data in mosquitoes unequivocally indicate, however, that the expression of

Table 2. In vitro (metabolism and/or depletion) and in vivo (*An. gambiae* and *D. melanogaster*) functional validation of *An. gambiae* *Cyp6m2*, *Cyp6p3*, and *Gste2* genes

| Class, insecticide, and gene | In vitro | <i>An. gambiae</i> (this study) | <i>D. melanogaster</i> |
|------------------------------|--|---------------------------------|------------------------|
| Pyrethroids | | | |
| Permethrin | | | |
| <i>Cyp6m2</i> | ✓ (11),* (19) [†] | ✓ | ✓ (15) |
| <i>Cyp6p3</i> | ✓ (10),* (19) [†] | ✓ | ✓ (15) |
| <i>Gste2</i> | N/A | ✗ | ✗ (20) |
| Deltamethrin | | | |
| <i>Cyp6m2</i> | ✓ (11),* (19) [†] | ✓ | ✓ (15) |
| <i>Cyp6p3</i> | ✓ (10),* (19) [†] | ✓ | ✓ (15) |
| <i>Gste2</i> | N/A | ✗ | N/A |
| Organochlorines | | | |
| DDT | | | |
| <i>Cyp6m2</i> | ✗ (19), [†] ✓ (12)*, [‡] | ✗ | ✓ (15) |
| <i>Cyp6p3</i> | ✗ (19) [†] | ✗ | N/A |
| <i>Gste2</i> | ✓ (9, 13)* | ✓ | ✓ (13, 20) |
| Carbamates | | | |
| Bendiocarb | | | |
| <i>Cyp6m2</i> | ✗ (15, 19) [†] | ✗ | ✓ (15) |
| <i>Cyp6p3</i> | ✓ (15, 19) [†] | ✓ | ✓ (15) |
| <i>Gste2</i> | N/A | ✗ | N/A |
| Organophosphates | | | |
| Malathion | | | |
| <i>Cyp6m2</i> | ✓ (28),* (19) [†] | ✓ | N/A |
| <i>Cyp6p3</i> | ✓ (19) [†] | ✓ | N/A |
| <i>Gste2</i> | N/A | ✗ | N/A |
| Fenitrothion | | | |
| <i>Cyp6m2</i> | ✓ (19) [†] | N/A | N/A |
| <i>Cyp6p3</i> | ✓ (19) [†] | N/A | N/A |
| <i>Gste2</i> | N/A | ✓ | N/A |

Presence (✓) or absence (×) of in vitro activity or in vivo WHO-defined insecticide resistance (*An. gambiae*) or increased insecticide tolerance (*D. melanogaster*). N/A, no information available.

*Data in reference indicated that metabolism of substrate had been observed through the production of metabolites.

[†]Data in reference indicated that substrate had been depleted with no direct evidence of metabolite production.

[‡]In the presence of added cholate.

single *Cyp6* genes can confer resistance to different pyrethroids and that *Cyp6p3* overexpression confers cross-resistance to prominent representatives of at least 2 classes of public health insecticides.

In contrast to our *Cyp6* studies, increased *An. gambiae* *Gste2* (*AgGste2*) expression generates clear DDT resistance, while resistance to bendiocarb and pyrethroids was not observed. These phenotypes again validate predictions from the DDT activity observed in vitro for recombinant AgGSTe2 (9, 13) as well as the increased DDT tolerance (13) and lack of pyrethroid tolerance (20) observed when overexpressed in *D. melanogaster*. The corresponding in vitro data for AgGSTe2 activity against bendiocarb and pyrethroids have not been reported, and here, bendiocarb resistance has been examined in vivo following *Gste2* overexpression.

Although DDT tolerance was also observed in *D. melanogaster* overexpressing the orthologous *AfGste2* (16, 18), conflicting results were reported about activity toward pyrethroids. For example, recombinant AfGSTe2 depleted permethrin but not deltamethrin in vitro, yet *D. melanogaster* acquired increased tolerance to both insecticides when *AfGste2* was overexpressed (16, 18). RNAi analysis in deltamethrin-resistant *Aedes aegypti* of *AaGste2* has also indicated a role in pyrethroid resistance (29). It is possible that the variation observed in resistance profiling is due to intrinsic differences in the activity of GSTe2s derived from the different mosquito species. In this context, it has been speculated that the predominant pyrethroid detoxification role of GSTs in some insects is sequestration or protection against oxidative stress rather than direct metabolism (30). Our results show that even high levels of *AgGste2* overexpression do not confer WHO diagnostic levels of resistance to this class of insecticides in isolation. It is feasible that the level of glutathione cofactor is limiting in the mosquito; however, this seems unlikely, since the recombinant enzyme does not show pyrethroid metabolizing activity, even in the presence of excess glutathione (20). It is also possible that *AgGste2* may need to work in concert with other genes that are not up-regulated in the sensitive genetic background of the *An. gambiae* transgenic lines to produce a pyrethroid resistance phenotype. Future work will test this hypothesis by coexpression of other UAS-regulated detoxification genes using the Ubi-A10GAL4:UAS-e2 (integration) line. Although beyond the scope of this work, this mosquito line expresses GAL4 and GSTe2 and can be crossed with other UAS lines to provide coexpression with other detoxification enzymes to examine additive or synergistic interactions.

Although GSTs have been associated with OP metabolism through biochemical studies (7), we report evidence that the expression of a single gene can provide OP resistance in mosquitoes. The high resistance shown toward fenitrothion by *Gste2*-overexpressing *An. gambiae* is intriguing. It is currently unclear if GSTe2 detoxifies fenitrothion by sequestration, by free radical protection, or directly through conjugation/modification. Evidence from early studies (31) suggests that *Anopheles* GST activity is associated with the conversion of fenitrothion to the nontoxic metabolite desmethyl fenitrooxon through an oxidized intermediate. Similar analysis in the *Gste2*-overexpressing lines would clarify which of these mechanisms is involved. Further investigation is also needed on the OP malathion, for which we report suspected resistance when *Gste2* is overexpressed.

We have also demonstrated that *Cyp6* overexpression increases susceptibility to malathion as well as conferring permethrin resistance, which may have direct implications on insecticide management, especially if replicated with other OPs that may be used for *Anopheles* control (32). Such sensitivity profiles are readily explained by the bioactivation of malathion to its more toxic metabolite malaaxon (33) by a P450-mediated mechanism (28). Direct evidence of activation has been shown by mass spectrometry analysis of in vitro CYP6M2 activity against malathion, which identified a major metabolite of 315 Da, corresponding to malaaxon (28). Here, we provide direct in vivo evidence that CYP6 enzymes can confer negative cross-resistance. Furthermore,

there seems to be substrate specificity in the alternative P450-mediated reactions, since we observed higher mortality when assayed against *Cyp6m2* overexpression compared with *Cyp6p3*. This may suggest that *Cyp6m2* favors the higher steady-state production of the toxic intermediate compared with *Cyp6p3*.

Malathion activation by *Cyp6m2* is also supported by recent evidence provided by Ingham et al. (34), who found that knockdown of the transcription factor Maf-S results in increased survival following malathion exposure. One of the P450s down-regulated by Maf-S knockdown was *Cyp6m2*, whereas *Cyp6p3* transcription was not modified. Taken together, the results provide experimental evidence to support the use of OPs and potentially, other proinsecticides activated by CYP6 enzymes for *Anopheles* control in areas where pyrethroid resistance is also conferred by detoxification by the same enzyme(s). One such strategy involves combining the use of pyrethroid-based bed nets with OP-based residual wall spraying or impregnated hangings (32). This takes advantage of the additive effect of the 2 classes of insecticides while sensitizing *Cyp6*-based pyrethroid-resistant mosquitoes to malathion (35). In conjunction with recombinant enzyme assays, the modified mosquitoes described may thus become valuable tools to assess the susceptibility of public health proinsecticides (for example, chlorfenapyr [36]) to activation and detoxification by xenobiotic metabolizing P450 genes in *Anopheles*.

When validating resistance phenotypes conferred by transgenic overexpression, the spatial pattern of overexpression can give clues to the identity of key tissues of detoxification. The expression driven by Ubi-A10 is spread over multiple tissues, which makes it impossible to pinpoint which tissues are particularly important for generating the resistance phenotype. Here, we directly investigated the involvement of the midgut and oenocytes in conferring P450-mediated resistance. Critically, we did not observe clear resistance to any insecticide class when either *Cyp6m2* or *Cyp6p3* was specifically expressed in either of these tissues, despite achieving highly enriched expression and the knowledge that oenocytes and the midgut express abundant P450 coenzyme CPR (26). Furthermore, since our previous expression profiling of the Ubi-A10 driver indicated lack of expression in Malpighian tubules (23), yet resistance to multiple insecticides was observed with this driver, it would seem that the insecticides tested are not predominately metabolized in the Malpighian tubules either, and other unidentified tissues may be critical, alone or in combination, for detoxification. As described earlier, some evidence of tissue specificity of P450s associated with insecticide resistance has been derived from transcriptomic analysis of crude dissections of tissues and body segments from pyrethroid-resistant and -sensitive strains (27). This study indicated that *Cyp6p3* is more highly expressed in the midgut of the resistant strain, whereas *Cyp6m2* has a broader up-regulation in midgut, Malpighian tubules, and the abdomen (integument, fat body, and ovaries). The relevance of elevated *Cyp6p3* levels in the midgut of the examined resistant strain is difficult to reconcile with the lack of a resistance phenotype when the same gene is overexpressed in this tissue with the GAL4/UAS system.

Previous *D. melanogaster* studies have shown that overexpression using drivers active in multiple tissues, such as actin5C-GAL4 (14–18) or tubulin-GAL4 (20), is generally needed to modify resistance. Nevertheless, there are few examples in which tissue-specific drivers have been used to validate *Cyp6* gene-based resistance in *D. melanogaster*. Yang et al. (37) demonstrated the central role of Malpighian tubules for *DmCyp6g1*-mediated DDT resistance, while Zhu et al. (38) demonstrated the importance of neuronal expression to provide deltamethrin resistance in *D. melanogaster* expressing *Tribolium castaneum* *Cyp6bq9*. Even in this latter analysis, however, the neuronal driver showed leaky expression in other tissues, leading to the possibility that the observed phenotype results from expression in multiple tissues. Further work in *D. melanogaster* with alternative tissue-specific drivers could be exploited to provide insight into the involvement of particular tissues in *Cyp6*-mediated

resistance. When similar tools become available in mosquitoes, a more definitive answer to which specific tissues are involved in the detoxification of insecticides in *An. gambiae* can be provided.

Conclusions

This work reports on functional analysis of mosquito insecticide resistance genes conducted in transgenic *An. gambiae*. The mosquitoes generated are resistant, in a solely metabolism-based manner, to at least 1 representative insecticide from the major classes used in public health and are, therefore, useful in prescreens of new and repurposed active compounds, including insecticides, proinsecticides, synergists, and sterilizing agents. The lines can also be used in combination with strains carrying genome-edited target sites (e.g., Kdr and Ace-1R) to examine the additive or synergistic effects of multiple resistance mechanisms. Similarly, it is possible to use the integration line carrying both Ubi-GAL4 and UAS-*Gste2* to cross with other UAS detoxification genes to analyze metabolic interactions: for example, combining phases I and II metabolism. In addition, the Ubi-A10 driver is active in larval stages (23) and can thus be used to examine gene function in immature stages.

Importantly, for future work, there is growing evidence on the involvement in resistance of genes that are very difficult to test either in vitro, due to the lack of appropriate assays, or in *D. melanogaster*, since interacting partner proteins may be different or absent. These include genes coding for cuticle components (39), transcription factors (34), and other binding proteins (e.g., hexamerins and α -crystallins [21]), for which current transgenic tools, including GAL4/UAS, make *An. gambiae* the most relevant option for functional genetic analysis.

Materials and Methods

Plasmid Construction. Responder plasmids were designed for the expression of the *An. gambiae* genes *Cyp6m2* (AGAP008212), *Cyp6p3* (AGAP002865), or *Gste2* (AGAP009194) under the regulation of the UAS and carried a YFP marker gene regulated by the 3xP3 promoter. The coding sequences of *Cyp6m2* (1,500 bp), derived from the susceptible strain Kisumu, were amplified from PB13:CYP6M2 (11) using primers M2fw and M2rv (SI Appendix, Table S1). The coding sequence of *Cyp6p3* was obtained by amplifying a 193-bp fragment from Kisumu complementary DNA using primers P3fw1 and P3rv1 (SI Appendix, Table S1) and a 1,362-bp fragment from pCW:17 α -*Cyp6p3* (10) using primers P3fw2 and P3rv2 (SI Appendix, Table S1). P3fw1 and P3rv2 were then used to join the 2 fragments and obtain the 1,530-bp full-length *Cyp6p3* coding sequence. The 666-bp *Gste2-114T* coding sequence derived from the DDT-resistant strain ZAN/U was amplified from the K1B plasmid (13) using primers *Gste2k1bfor* and *Gste2k1brev* (SI Appendix, Table S1). All coding sequences were cloned into the YFP-marked responder plasmid pSL*attB:YFP:Gyp:UAS14i:Gyp:attB (24) downstream of the UAS using EcoRV/XhoI (*Cyp6*) or EcoRI/NcoI (*Gste2*).

Creation of UAS Responder Lines by PhiC31-Mediated Cassette Exchange. For creating responder lines carrying *Cyp6* genes, embryos of the docking line A11 (24), which carries 2 inverted *attP* sites and is marked with 3xP3-driven CFP, were microinjected with 350 ng/ μ L of the responder plasmid and 150 ng/ μ L of the integrase helper plasmid pKC40 encoding the phiC31 integrase (40) as described in Pondeville et al. (41). The same protocol was followed to create the *Gste2* responder line using embryos of the docking line Ubi-A10 (23), which carries 2 inverted *attP* sites and is marked with 3xP3-driven CFP. Emerging F_0 was pooled into sex-specific founder cages and outcrossed with wild-type G3s. F_1 progenies were screened for the expression of YFP (cassette exchange) and CFP/YFP (cassette integration) in the eyes and nerve cord. Orientation check to assess the direction of cassette exchange was performed on F_1 YFP-positive individuals or on the F_2 progeny deriving from single YFP-positive individuals. This was carried out by PCR using alternative combinations of 4 primers designed to give a product only in 1 of the orientations: Two pairs were diagnostic for orientation A, and two pairs were diagnostic for orientation B. Mosquito DNA with an insertion in orientation A gives products only with PiggyBac-R2 + Red-seq4R (PCR1) and gene-specific primers M2intFW or P3intFW or *Gste2_v1* + ITRL1R (PCR2). Mosquito DNA with an insertion in orientation B gives products only with PiggyBac-R2 + gene specific primers M2intFW or P3intFW or *Gste2_v2* (PCR3) and Red-seq4R + ITRL1R (PCR4). All definitive responder lines were created from individuals showing orientation of insertion A, which was chosen for consistency with previous RMCE lines created in this laboratory. Transformation

efficiencies were calculated as the number of independent transgenic events (exchanges or integrations) over the number of surviving F_0 adults.

Driver Lines and GAL4 \times UAS Crosses. Crosses for ubiquitous expression were established between the CFP-marked driver Ubi-A10 (23) and individuals of the responder lines marked with YFP. To obtain tissue-localized expression, dsRed-marked drivers specific for expression in the midgut (GAL4-mid) (22) or in the oenocytes (GAL4-*oeno*) (24) were used. Responder lines were kept as a mix of homozygous and heterozygous individuals so as to obtain GAL4/+ progeny to be used as transgenic blank controls.

Cyp6 Gene Expression Analysis. To quantify *Cyp6* gene expression in GAL4/UAS and GAL4/+ individuals, total RNA was harvested from pools of 2- to 5-d-old whole adults and their relevant dissected body part (midgut or abdomen cuticle). The adult tissues remaining after dissection constituted the carcass. Three biological replicates consisting of 5 mosquitoes (or body parts) each were collected from each mosquito population. RNA extraction was performed using the TRI Reagent protocol (Sigma). To remove genomic DNA contamination, samples were treated with the Turbo DNA-Free kit (Ambion). RNA was then reverse transcribed using the SuperScript III First-Strand Synthesis System (Life Technologies) following the oligo(dT) reaction protocol. qRT-PCR reactions were set up using 1 \times Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) and primers qM2fw and qM2rv for quantification of *Cyp6m2* and qP3fw and qP3sub for *Cyp6p3* (15) (SI Appendix, Table S1). The qP3sub primer bears a nucleotide substitution (A11G) to conform its sequence to that of the G3 strain template. Two housekeeping genes, the ribosomal protein S7 (AGAP010592) and the ribosomal protein L40/Ubiquitin (AGAP007927), were also quantified using primers qS7fw, qS7rv, qUBfw, and qUBrv (42) (SI Appendix, Table S1). *Cyp6* transcription data obtained by qRT-PCR were analyzed using the $\Delta\Delta$ cycle threshold (C_t) method as described in SI Appendix, Supplementary Method. Gene expression analysis was not performed to assess up-regulation of the *Gste2* transcript.

CYP6 and GSTE2 Protein Expression Analysis. To detect protein expression in GAL4/UAS and GAL4/+ individuals, total protein extracts were obtained from whole 2- to 5-d-old female adults and their dissected body parts. Whole mosquitoes and dissected body parts were directly homogenized in 1 \times Laemmli buffer (BioRad) containing 5% 2-mercaptoethanol and 1 \times protease inhibitors (cOmplete EDTA-free Protease Inhibitor Mixture; Sigma), incubated at 95 $^{\circ}$ C for 10 min, and then, centrifuged at 13,000 rpm for 5 min. Volumes of protein extract were separated by sodium dodecylsulphate/polyacrylamide gel electrophoresis to give the number of whole mosquito or body part equivalents as indicated.

Protein extracts equivalent to 1/3 of a mosquito or its body part were analyzed to detect CYP6 expression driven by tissue-specific drivers with the exception of midgut samples, for which 2 whole midguts were analyzed. The higher amount of midgut sample was required to visualize signal of the α -tubulin loading control. The equivalent of 1/10 of a single female mosquito was used to assess expression driven by ubiquitous drivers. CYP6s were probed using primary affinity-purified polyclonal peptide antibodies produced in rabbit against CYP6M2 or CYP6P3 (gifts from M. Paine Liverpool School of Tropical Medicine (LSTM), Liverpool, UK), while GSTE2 was probed with anti-GSTE2-28 rabbit primary antibodies (9). Secondary antibodies were anti-rabbit horseradish peroxidase (HRP)-tagged immunoglobulins G (IgG) (Bethyl Laboratories). Relative protein loading and blotting efficacy was verified by reprobing stripped membranes (Restore ThermoFisher) using primary mouse anti- α -tubulin antibodies (Sigma) and secondary goat anti-mouse HRP IgG antibodies (Abcam). Signal detection was carried out using SuperSignal West Dura Extended Duration Substrate (Life Technologies).

Assessment of Susceptibility to Insecticides. Susceptibility to insecticides was assessed in mosquitoes overexpressing *Cyp6* genes using the WHO tube bioassay (25). Pools of 20 to 25 GAL4/UAS and GAL4/+ adult female mosquitoes were exposed 2 to 5 d postemergence to standard discriminating doses of insecticides—0.75% permethrin, 0.05% deltamethrin, 0.1% bendiocarb, and 4% DDT—for 60 min, and mortality rates were assessed after a 24-h recovery period. For mosquitoes expressing *Cyp6* genes in the midgut or oenocytes, a modified version of the standard WHO test was also performed, reducing the exposure time to 20 min (26). For assessing susceptibility to 5% malathion in mosquitoes overexpressing *Cyp6* genes, the exposure time was decreased to 25 min. Mosquitoes overexpressing *Gste2* were additionally tested for 1% fenitrothion using the recommended 2-h exposure time; 1 to 4 biological replicates were performed for each insecticide tested. A total of 2 to 8 technical replicate tubes were tested for each population. Welch's *t* test was performed to determine statistical differences between mortality rates in GAL4/UAS and GAL4/+. Details on replicate numbers of insecticide bioassay

experiments and statistical analysis are reported in *SI Appendix, Table S2*. All statistics were calculated using GraphPad Prism version 8.2.1 (GraphPad Software; <https://www.graphpad.com/>).

Data Availability. All extant transgenic lines, primary antibodies, and plasmids described will be provided by G.J.L. on request. The raw bioassay data can be accessed at Figshare (43).

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